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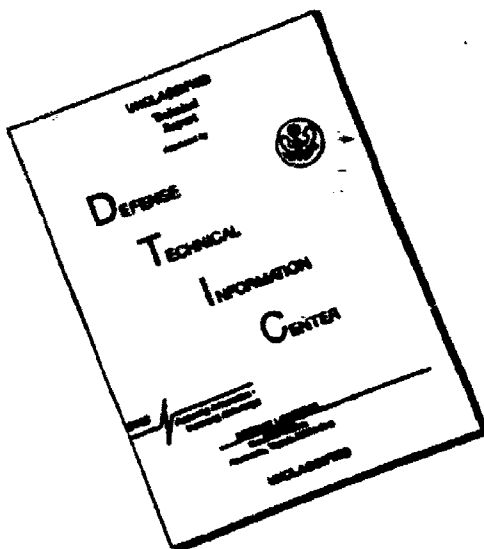
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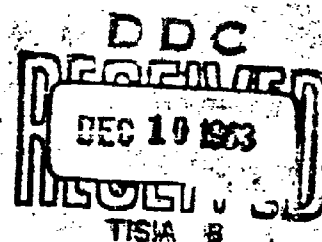
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# TECHNICAL MANUSCRIPT 108

## EFFECT OF GUANIDINIUM SALTS ON THE TOXICITY OF BOTULINUM TOXIN

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UNITED STATES ARMY  
BIOLOGICAL LABORATORIES  
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EFFECT OF GUANIDINIUM SALTS ON THE TOXICITY  
OF BOTULINUM TOXIN

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# ABSTRACT

The effect of guanidinium salts on the stability of botulinum toxin and the mechanisms through which denaturation by these salts occurs is described. Some salts are effective in reducing toxicity at low concentrations; in others, toxicity is retained even in saturated solution. The nature of the interaction is complex, involving more than a change in folding or conformation. The hypothesis that salt solutions with high thermodynamic water activity labilize the hydrogen-bonded structure of the protein is shown to be invalid. Also shown to be inapplicable is the hypothesis that a direct effect of the anion on the guanidinium cation leads to a reduction of its thermodynamic activity and ability to break hydrogen bonds. The protective mechanism appears to operate via binding of anions across clusters of cationic sites on the charged protein to preserve spatial configurations and charge distributions.



## EFFECT OF GUANIDINIUM SALTS ON THE TOXICITY OF BOTULINUM TOXIN

The nature of the mechanism through which urea and guanidinium salts denature proteins has been the subject of continuing investigation, and most recently a solubilizing action on hydrophobic groups has been proposed as the responsible property.<sup>1,2</sup> In these studies, guanidinium chloride has been the salt almost universally selected. There is evidence, however, that all guanidinium salts are not equally effective. Greenstein reported that, although guanidinium chloride liberated titratable sulfhydryl groups from ovalbumin, the sulfate, acetate, and carbonate salts did not. This difference led us to examine the effect of a series of guanidinium salts of various anions upon the biological activity and structure of botulinum toxin, type A. We find that a specific anion effect is indeed involved in the action of guanidinium salts.

The salts were prepared from guanidinium carbonate and the desired acid by double displacement and their purity was checked by micro-Kjeldahl. The inactivations were carried out by adding 0.10 milliliter of crystalline botulinum toxin, type A, at a concentration of four micrograms per milliliter in pH 3.8, 0.05 M acetate buffer to each of a series of tubes containing 10.0 milliliters of a guanidine salt of known concentration, mixing gently, and allowing the mixture to stand overnight at room temperature. The mixtures were at pH 6.5 to 7, on the alkaline side of the protein isoelectric point. Toxicities were determined by bioassay.<sup>4</sup> Controls of protein-free guanidinium salts exhibited no toxicity to mice\* at the dilutions used for the toxin assay.

Table I shows the inactivation of botulinum toxin by several salts, including guanidinium chloride and urea as reference comparisons. The most striking observation is the wide variation obtained, a difference of some fortyfold at the extremes. More than half of the salts tested were more effective denaturants than guanidinium chloride; on the other hand, complete stability of the toxin was found in saturated solutions of several others. No explanation is presently available for the difference between meso- and D,L-tartrate and between the two phosphates that might be expected to equilibrate to nearly identical composition.

The nature of the interaction is complex, involving much more than a change in folding or conformation. In 1 M guanidinium chloride, the toxin retained its toxicity and was homogeneous in the ultracentrifuge with an unchanged value of  $s_{20,w}$ . In 2 M guanidinium chloride the toxicity was destroyed and three peaks were observed in the ultracentrifuge. In 4 M guanidinium sulfate, on the other hand, toxicity was retained and only one peak with an unaltered sedimentation constant was found in the ultracentrifuge.

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\* In conducting the research reported herein the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

TABLE I. INACTIVATION OF BOTULINUM TOXIN BY GUANIDINIUM SALTS

Anion of Guanidinium Salt	Concentration for 50% Inhibition, M
Benzoate	0.1
Meso-Tartrate	<1
Phthalate	<1
Thiocyanate	0.75
Monohydrogenphosphate ( $\text{HPO}_4^-$ )	0.8 <sup>a</sup>
Fluoride	0.9
Glutarate	0.9
Acetate	0.9
Malonate	0.9
Citrate	1.25
Fumarate	1.4
Chloride	2.0
D,L-Tartrate	2.25
Malate	3.3
Succinate	3.3
Oxalate	>3 <sup>a</sup>
Dihydrogenphosphate ( $\text{H}_2\text{PO}_4^-$ )	>3.6 <sup>a</sup>
Sulfate	>4 <sup>a</sup>
Urea	6

a. Saturated solution.

Although some of the salts are out of line, notably citrate, a reasonable correlation was observed when the salt concentration causing 50 per cent reduction in toxicity was plotted against available values of the free energy or enthalpy of hydration of the anions (benzoate, thiocyanate, acetate, chloride, and succinate) or against the free energy of formation of the ions from the elements. All of these parameters are known to be linearly proportional to the lyotropic numbers.<sup>6</sup> Lyotropic action has been pictured<sup>6</sup> "as a matter of competition between the dissolved salt and the macromolecular component towards water." According to this mechanism, an alteration of the hydration state at a hydrophilic junction point in a protein will result in a free energy change, the sign and magnitude of which will govern the physico-chemical or biological changes associated with the lyotropic series. A similar concept was employed by Harrington and Schellman<sup>7</sup> to explain the ability of certain salts (e.g.  $\text{LiBr}$ ) to enhance protein stability. They proposed that solutions of salts with high activity coefficients, and correspondingly greatly reduced water activity, tend to cause a maximum number of intramolecular hydrogen bonds to form in a protein. Conversely, salts with low activity coefficients and a high water activity should tend to labilize the hydrogen-bonded structure of a protein and decrease its stability. This hypothesis

is inapplicable in our case. Indeed, just the opposite of the predicted result was obtained, botulinum toxin being more stable in solutions of guanidinium salts with low activity coefficients.

An alternative and more satisfactory explanation for the specific anion effect is based on anion binding to the protein molecule. Such binding is voluminously documented; Scatchard's studies<sup>9</sup> with serum albumin are particularly pertinent. The anions of a series of salts with a common cation were shown to bind according to a lyotropic series. Boyer *et al.*<sup>9,10</sup> showed that anion binding could increase protein stability. Lengthening the carbon chain from acetate to caprylate progressively augmented the stability of serum albumin against thermal, urea, and guanidine denaturation. Their data demonstrated that the stabilization was a function of the size and structure of the anion and the amount bound. However, it should be emphasized that there is no *a priori* reason for anion binding to protect a protein; indeed, caprylate was shown to labilize several proteins. Benzoate has a similar effect on botulinum toxin; the sodium salt inactivated at the same concentration as the guanidinium salt. The mechanism of protection afforded botulinum toxin by anion binding is obviously then only conjecture. Cationic side chains certainly participate in this binding, either singly<sup>11</sup> or in cluster,<sup>12</sup> and it is perhaps significant that charged lysine groups are essential for the retention of toxicity.<sup>13</sup> The anion may preserve the spatial charge interrelationships of the active toxin.

One additional explanation may be invoked to explain the present data. This is a direct effect of the anion upon the guanidinium cation leading to a reduction in its thermodynamic activity. Reduced activity coefficients in concentrated solutions are attributed either to the formation of an ion-pair or a complex ion.<sup>14</sup> Aside from the fact that there is no reason to assume these forms are inactive as denaturants, we believe that this mechanism is not operative. Examination of tables of activity coefficients of the sodium and potassium salts of some of these anions, (e.g. thiocyanate, fluoride, and acetate) reveals that the differences are not sufficient to account for the effects observed. Greenstein's observation<sup>7</sup> that guanidinium acetate did not denature ovalbumin provides further evidence, since sodium and potassium acetate have higher activity coefficients than the respective chlorides.

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